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Binding and Antigen Presentation of Ceramide-containing Glycolipids by Soluble Mouse and Human CD1d Molecules

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Summary

We have purified soluble mouse and human CD1d molecules to assess the structural requirements for lipid antigen presentation by CD1. Plate-bound CD1d molecules from either species can present the glycolipid α-galactosyl ceramide (α-GalCer) to mouse natural killer T cells, formally demonstrating both the in vitro formation of antigenic complexes, and the presentation of α-GalCer by these two CD1d molecules. Using surface plasmon resonance, we show that at neutral pH, mouse CD1 and human CD1d bind to immobilized α-GalCer, unlike human CD1b, which requires acidic pH for lipid antigen binding. The CD1d molecules can also bind both to the nonantigenic β-GalCer and to phosphatidylethanolamine, indicating that diverse lipids can bind to CD1d. These studies provide the first quantitative analysis of monomeric lipid antigen–CD1 interactions, and they demonstrate that the orientation of the galactose, or even the nature of the polar head group, are likely to be more important for T cell receptor contact than CD1d binding.

Key words: antigen presentation • CD1 • glycolipid • binding assay

The CD1 proteins are cell surface glycoproteins structurally similar to MHC class I molecules, in that they consist of a 43–49-kD heavy chain noncovalently associated with a β2-microglobulin (β2m)[1] light chain (1). There are two highly similar CD1 genes in mice, *CD1D1* and *CD1D2* (2). The human CD1 gene family contains five members, *CD1A* through *CD1E*, which fall into two groups on the basis of predicted amino acid sequence similarity (3). The *CD1A*, *-B*, and *-C* genes comprise group I. The human, mouse, rat, and rabbit *CD1D* genes comprise the second group (3). The CD1 molecules are distinguished from the MHC-encoded classical class I antigens by several properties, including their nonpolymorphic nature, their lack of a requirement for a functional transporter associated with antigen processing (TAP) for cell surface expression (4–8), their endosomal localization (9–12), and their restricted tissue distribution (13).

CD1 molecules probably have a unique role in the immune system due to their ability to present lipid antigens to T cells. Human group I CD1b and CD1c molecules can present several types of lipoglycan antigens from *Mycobacteria* species (4, 5, 14–17). Recently, mouse (m)CD1 and human (h)CD1d have been shown to present α-galactosyl ceramide (α-GalCer) to NK T lymphocytes, providing the first evidence for lipoglycan antigen presentation by group II or CD1d molecules (18–23). NK T cells are characterized by expression of activating and inhibiting receptors found on NK cells, a relatively invariant TCR, and the ability to rapidly produce large amounts of cytokines (24, 25). In the presence of α-GalCer, the autoreactivity of NK T cells for CD1d molecules can be greatly augmented, although β-GalCer is not antigenic (18–23). The expression of the invariant TCR characteristic of the NK T lymphocyte subpopulation, namely Vα14 along with any one of several Vβ genes in mouse, or Vα24 plus Vβ11 in humans, is required for a CD1d-mediated α-GalCer response (19, 20). Interestingly, human NK T cells can recognize α-GalCer presented by mCD1, whereas mouse NK T cells recognize α-GalCer plus hCD1d (20). This high degree of conservation is consistent with the fundamental importance of NK T cell specificity.

Although the presentation of α-GalCer by CD1d molecules has been well established, there are no reports on the biochemistry of the interaction of ceramide-containing lipids with CD1. Indeed, a phospholipid related to glycophosphati-

[1]*Abbreviations used in this paper:* β2m, β2-microglobulin; GalCer, galactosyl ceramide; DPPE, dipalmitoyl phosphatidylethanolamine; GPI, glycophosphatidylinositol; HA, hemagglutinin; HRP, horseradish peroxidase; mCD1, mouse CD1.1; PEG, polyethylene glycol; RU, response unit(s); SPR, surface plasmon resonance; TAP, transporter associated with antigen processing.

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dylinositol (GPI) is the predominant ligand that was eluted from mCD1 expressed by mammalian cells (26), and recently, reactivity of some NK T cells for GPI has been demonstrated (27). Furthermore, the quantitative study of the binding of CD1 molecules to lipids has been hampered by the tendency of these compounds to form insoluble aggregates. Therefore, we have used purified, soluble mCD1 and hCD1d molecules to study the biochemistry of binding and presentation of α-GalCer and several other glycolipids. To examine monomeric CD1–lipid interactions, we used immobilized derivatives of the lipids, which should be free of aggregates or micelles. We also examined the binding of mCD1 to a peptide ligand (28) by the surface plasmon resonance (SPR), and the effects of mCD1-binding lipids on this interaction with peptide.

Materials and Methods

Chemicals. The syntheses of biotinylated α- and β-GalCer (biotin–α-GalCer and biotin–β-GalCer) (see Fig. 3 A) have been described elsewhere (29). After synthesis, the biotinylated compounds were purified by silica gel column, which completely removed the biotinylating reagent (29). The purity of biotinylated α- and β-GalCer compounds was verified by mass spectrometry. No unbiotinylated galactosyl ceramide was detectable in the final preparation of the compound (29). *N*-(biotinoyl)dipalmitoyl-l-α-phosphatidylethanolamine (EZ-Link™ biotin-DPPE; see Fig. 5 A) was purchased from Pierce Chemical Co. Gangliosides GM1 and GD1a were purchased from Calbiochem. Hexasaccharide disialyllacto-*N*-tetraose (α-Neu5Ac-[2→3]-β-Gal-[1→3]-(α-Neu5Ac-[2→6])-β-GlcNac-[1→3]-β-Gal-[1→4]-Glc) was purchased from Sigma Chemical Co. Polyethylene glycol $(PEG)_{2000}$ ceramide was purchased from Northern Lipids, Inc. P99 peptide (YEHDFHHIREWGNHWKNCLAVM) and NH_2 terminus biotinylated P99 peptide were synthesized at Research Genetics, Inc.

Gene Constructs and Transfectants. For expression of soluble hCD1d molecules in insect cells, we generated the plasmid hCD1d-pRmHa-3, which encodes a truncated form of the hCD1d cDNA containing 32 bp of 5′ untranslated region, the signal peptide for insertion into the endoplasmic reticulum, and the cell surface α1–α3 domains. The hCD1d coding sequence was amplified using PCR from a plasmid encoding hCD1d (a gift of Dr. Steven Balk, Beth Israel Hospital, Boston, MA). Oligonucleotides corresponding to the 5′ untranslated region upstream of the hCD1d AUG start codon (TTGCAGCCATGGAGGTCCCCACG) and the distal end of the cytoplasmic domain (CGGGATCCCCAGTAGAGGACGATGTCCTG) introduced 5′ NcoI and 3′ BamH1 restriction enzyme sites, which allowed for cloning of the truncated cDNA into a modified version of the insect cell expression vector, pRmHa-3 (30; a gift of Dr. A. Raúl Castaño, Instituto de Salud Carlos III, Madrid, Spain). Following the BglII cloning site in this vector are sequences encoding 2 protein tags; the 10 amino acid sequence, YPYDVPDYAS, derived from influenza hemagglutinin (HA), which is recognized by the 7F11 mAb; and a 6-histidine tag for protein purification on Ni-NTA agarose (Qiagen). *Drosophila melanogaster* cells in tissue culture were cotransfected with 15 μg of hCD1d-pRmHa-3, 15 μg of hβ2m-pRmHa-3, encoding hβ2m, and 1 μg of pUChsneo by the calcium phosphate method and selected with 1–1.5 mg/ml of G418 for 2 mo. The hβ2m-pRmHa construct was a gift from Dr. Jeffrey E. Miller (IVS Technologies LLC, Carlsbad, CA).

Cell Lines. Cultures of parental and mCD1- and hCD1d-transfected *D. melanogaster* embryonic SC2 cells were carried out as described previously (31). For the culture of transfected insect tissue culture cells, the medium was supplemented with 1 mg/ml G418 (Sigma Chemical Co.). The mCD1-transfected *D. melanogaster* cells and untransfected parental insect cell lines were a gift of Dr. A. Raúl Castaño. The mCD1-transfected A20 cell lines have been described previously (32).

Antibodies. Antibodies used for ELISA were 7F11 (mouse IgG_{2b}) for detection of HA epitope–tagged polypeptides; BBM.1 for hβ2m (33), biotinylated 1B1 (rat IgG_{2b}) for mCD1 (32), and NOR3.2 (mouse IgG_1) for hCD1d (34). Horseradish peroxidase (HRP)-conjugated goat anti–mouse Ig (Caltag Labs) and HRP-conjugated streptavidin (Jackson ImmunoResearch Labs) were used as secondary reagents in ELISA.

Purification of Soluble CD1d. 3–10 liters of insect cells, stably cotransfected with the CD1d- and β2m-encoding plasmids, were induced with 0.7 mM $CuSO_4$ for 3 d to transcribe the metallothionein promoter. After induction, cell supernatant was concentrated and dialyzed against 0.15 M sodium phosphate buffer (pH 7.4) using an Amicon Y1S10 Spiral Wound Membrane cartridge with a 10-kD mol wt cut-off (Millipore). Soluble CD1d–β2m complexes were first affinity-purified on Ni-NTA agarose column (1 ml bed volume; Qiagen) and then FPLC purified with Mono Q anion-exchange column (1 ml bed volume; Amersham Pharmacia Biotech). Gel filtration was performed in PBS on a Superdex 200 column (Amersham Pharmacia Biotech) to verify that purified protein did not form aggregates. Protein concentration was determined from the absorbency at 280 nm and by using the bicinchoninic acid protein assay reagent (Pierce Chemical Co.). Soluble mouse class I molecule H2-M3 was a gift of Drs. Santai Shen and Johann Deisenhofer (Howard Hughes Medical Institute, University of Texas, Southwestern Medical Center, Dallas, TX).

NK T Cell Hybridoma Stimulation. The Vα14Vβ8 NK T cell hybridomas N38-3C3 and N38-2C12 (a gift of Dr. Kyoko Hayakawa, Fox Chase Cancer Center, Philadelphia, PA) and DN3A4-1.2 (a gift of Dr. M. Bix, University of California San Francisco, San Francisco, CA) have been described previously (19). For the assays, T hybridoma cells were cultured for 16 h at 5×10^4 cells/well in the presence of mCD1-transfected A20 cells that had been prepulsed for 2 h with the indicated concentrations of glycolipids or with the vehicle DMSO. IL-2 release was evaluated in a sandwich ELISA using rat anti–mouse IL-2 mAbs (PharMingen), and the levels of cytokine release were evaluated using a recombinant IL-2 standard (PharMingen). For experiments in which α-GalCer was presented by immobilized CD1d proteins, 96-well tissue culture plates were coated for 2 h at 37°C with 1 μg/well of soluble protein that had been premixed with the indicated concentrations of lipid antigen in 100 μl PBS. Control wells were coated with protein only in the presence of DMSO (0.25–1% final concentration) or with lipids alone. After coating, the plate was washed five times in PBS and 5×10^4 hybridoma cells/well were added immediately. IL-2 release was measured after 16 h of culture. For competition experiments, gangliosides were dissolved in PBS and added to CD1d proteins before the addition of α-GalCer.

Surface Plasmon Resonance. A BIAcore X biosensor system was used for real time binding experiments, which were all done at room temperature. 1 mg/ml stock solutions in DMSO of biotin–α-GalCer or biotin–β-GalCer were diluted to 50 μg/ml into BIAcore flow buffer, Hepes-buffered saline (HBSt: 0.01 M Hepes, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.005% [vol/vol] surfactant P20), and captured onto a streptavidin sensor chip (BIAcore) at a flow rate of 5 μl/min, which yielded 200–400 re-

sponse units (RU) of immobilized ligand. Presence of immobilized biotin–α- and biotin–β-GalCer was additionally confirmed by binding of the galactose-specific lectins *Ricinus communis* agglutinin I (RCA I) and Isolectin B4 of *Griffonia simplicifolia* lectin I (GSL I isoform B4). 0.1 mg/ml stock solution of biotin-DPPE in DMSO was diluted to 2 μg/ml into BIAcore flow buffer for immobilization. 2 mg/ml stock solution of biotin-P99 in water was diluted to 20 μg/ml into BIAcore flow buffer for immobilization. For biotin-GalCer, kinetic studies were performed by injecting various concentrations of mCD1 or hCD1d in PBS or HBSt at 10–50 μl/min flow rate to measure the association phase, and washed with the same buffers and at the same flow rate to measure the dissociation phase. For biotin-DPPE, kinetic studies were done at 2 μl/min flow rate. Lipid-coupled surfaces were regenerated using 0.5–2-min pulses of PBS containing 0.05–1% Triton X-100 (Sigma Chemical Co.). For competition experiments, mixtures of protein with competitor lipids were preincubated at room temperature for 15–20 min before injection.

Estimation of Kinetic Rate Constants. Evaluation of the data was performed using the BIAevaluation software version 2.1 (BIAcore). For determination of the association rate constant, the association phase of the sensogram was fitted to a single exponential model and the k_{on} rate was determined from the plot of the observed association rate k_{obs} against protein concentration, according to the formula $k_{obs} = k_{on}C + k_{off}$. The dissociation phase of the sensogram was directly fitted to a single or double exponential model, and the contribution of each component to the overall dissociation was calculated.

Results

Purification of CD1d–β2m Complexes. To biochemically define the interaction of group II CD1 molecules with glycolipids, soluble hCD1d–hβ2m and mCD1–mβ2m heterodimers were produced in the *D. melanogaster*–derived SC2 cell line. This insect tissue culture system has previously been shown to produce functional human and mouse MHC class I, class II, and mCD1–mβ2m heterodimers (28, 35, 36). CD1d heterodimers were purified to 90–99% purity (Fig. 1 A) by nickel affinity chromatography and anion-exchange chromatography. The identity of the CD1d molecules produced was verified in ELISA in which specific mAbs were used to detect wells coated with the purified proteins (Fig. 1 B). The mAbs used include the hCD1d heavy chain–specific antibody NOR3.2, the hβ2m-specific antibody BBM.1, and the mCD1-specific antibody 1B1 (Fig. 1 B). The 7F11 antibody specific for the COOH-terminal influenza HA epitope tag on the soluble heavy chains recognized both CD1d proteins. Purified CD1d proteins were free of aggregates, as assessed by size exclusion FPLC chromatography (data not shown).

Immobilized CD1d Molecules Can Present α-GalCer to T Cells. Because of the tendency of lipids to interact nonspecifically with macromolecules, we wished to determine if physiologically relevant or antigenic lipid plus CD1d complexes could be formed in vitro. Soluble mCD1 and hCD1d molecules were preincubated with glycosphingolipids, and were used to coat the wells of microtiter plates that were subsequently cultured with N38-3C3 (hereafter 3C3), a $V\alpha14/V\beta8^+$ mouse NK T cell hybridoma (19). Previously, we demonstrated that some $V\alpha14^+$ mouse NK T cell hybridomas could recognize α-GalCer presented by either mCD1- or hCD1d-transfected APCs (19). In agreement with those results, we found that plate-bound mCD1 and plate-bound hCD1d could both present α-GalCer to the 3C3 T cell hybridoma, whereas plates coated with CD1d molecules without antigen, or with CD1d preincubated with β-GalCer, were not recognized (Fig. 2 A). Plates coated with the glycosphingolipid antigen alone also did not stimulate the hybridoma. Anti-mCD1 mAb 1B1 inhibited NK T cell recognition of α-GalCer presented by plate-bound mCD1, but had no effect on presentation by plate-bound hCD1d (Fig. 2 A). Thus, the T cell stimulation we observe is not due to the reuptake of α-GalCer and mCD1-mediated autopresentation by the hybridoma cells, which are weakly mCD1 positive.

The 3C3 hybridoma has a low level of autoreactivity to mCD1-transfected A20 cells in the absence of α-GalCer antigen (19, 20). Yet we did not observe any autoreactivity of this hybridoma to plates coated with soluble mCD1 that had not been preloaded with α-GalCer. The inability to react to plate-bound mCD1 suggests that the endogenous ligand present in soluble mCD1 molecules purified from *Drosophila* cells (Degano, M., and I. Wilson, personal com-

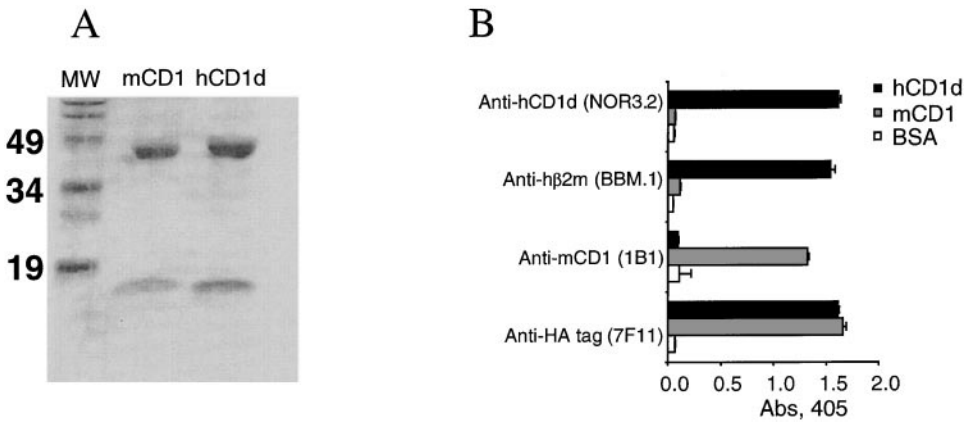

Figure 1. Purification of soluble CD1 proteins. (A) Coomassie blue–stained 12% SDS polyacrylamide gel showing FPLC-purified mCD1 and hCD1d proteins. Molecular weight (MW) standards are in the first lane with sizes (in kD) indicated. (B) ELISA confirming the identity of purified CD1d proteins. 200 ng of protein was coated per well; the blank wells were coated with BSA only. HRP-conjugated goat anti–mouse Ig was used as a secondary reagent to detect NOR3.2, BBM.1, and 7F11, and HRP-conjugated streptavidin was used to detect biotin-1B1. The values represent the average of duplicates of the absorbance values after addition of the HRP substrate.

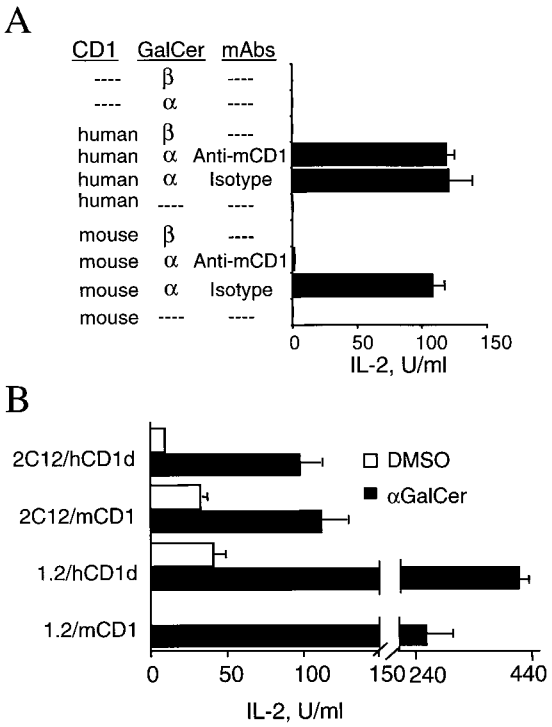

Figure 2. Immobilized purified CD1d molecules are able to present α-GalCer to NK T cells. (A) 1 μg/well mCD1 or hCD1d proteins preincubated with 100 ng of α-GalCer or β-GalCer and immobilized on a 96-well plate was used to stimulate the T cell hybridoma, 3C3. Some wells had antigen alone or CD1d protein alone. 15 μg/ml anti-mCD1 mAb 1B1 or isotype control antibody (Isotype) was added to some wells immediately before the addition of T cells. This experiment is representative of five independent experiments. (B) Stimulation of the 1.2 and 2C12 NK T cell hybridomas by immobilized mCD1 and hCD1d proteins (1 μg/well), either alone or with 50 ng/well of α-GalCer. This experiment is representative of five experiments.

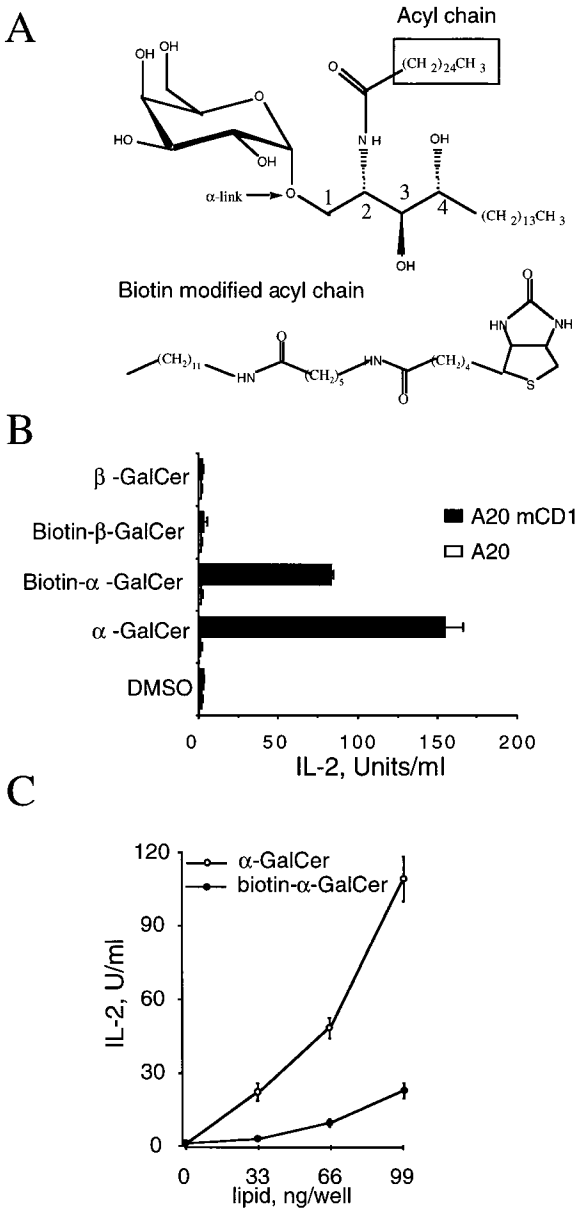

Figure 3. Biotin-modified α-GalCer is antigenic for NK T cells. (A) Antigen structures. α-GalCer is shown on the top, with the α-linkage of the sugar indicated by an arrow and the acyl chain of the ceramide moiety enclosed within a box. The position 1–4 carbons of the sphingosine are indicated. The biotin-modified acyl chain of biotin–α-GalCer and biotin–β-GalCer is shown below. (B) Biotin–α-GalCer can be recognized by NK T cells. mCD1-transfected or control, mock-transfected A20 cells were pulsed with 100 ng/ml of lipid antigen, or with 0.1% DMSO vehicle control, washed, and added to cultures of the 3C3 mouse NK T cell hybridoma for 16 h. IL-2 release was measured by ELISA. Data shown are from one experiment that was repeated five times. (C) Dose-dependent stimulation of the 3C3 hybridoma in response to increasing amounts of either α-GalCer or biotin–α-GalCer presented by 1 μg/well mCD1 immobilized on a microtiter plate. This experiment is representative of three independent experiments.

munication) may be different from the endogenous ligand(s) bound to mCD1 in A20 cells. Most interestingly, the pattern of autoreactivity of two other Vα14$^+$ NK T hybridomas, DN3A4-1.2 (hereafter 1.2) and N38-2C12 (hereafter 2C12), to mCD1 and hCD1d molecules was recapitulated in their autoreactivity to soluble CD1d proteins. The 1.2 hybridoma is strongly reactive to A20 hCD1d and weakly reactive to mCD1 transfectants, even without α-GalCer (19, 20). It also reacts to plates coated with soluble hCD1d (Fig. 2 B), producing significant amounts of IL-2 (40 U/ml in the experiment in Fig. 2 B; the IL-2 ELISA detection limit is 0.1 U/ml). Similarly, 2C12 has reactivity to both A20hCD1d and A20mCD1 transfectants without α-GalCer, and it produces IL-2 in response to plates coated with either CD1d protein (32 U/ml for mCD1 and 9 U/ml for hCD1d in the experiment shown in Fig. 2 B). While the reactivity of the 1.2 and 2C12 hybridomas to the insect cell–derived CD1d molecules is significant, this response can be enhanced by exogenously added α-GalCer, similar to the results with CD1d$^+$ APCs (19, 20). Finally, Vα14$^+$ mouse NK T cell hybridomas that express either Vβ7 (N38-2H4) or Vβ10 (DN3A4-1.4) TCR β chains also could recognize α-GalCer presented by immobilized mCD1 molecules, although they did not show reactivity to CD1d in the absence of this antigen (data not shown).

A Biotinylated α-GalCer Derivative Is Antigenic. We synthesized biotinylated versions of α-GalCer and the nonantigenic β-GalCer (29; Fig. 3 A) in order to develop a CD1d

binding assay in which the lipid could be immobilized onto a solid support with hydrophobic moieties exposed, thereby minimizing the problem of micelle or bilayer formation. The antigenicity of the biotinylated compounds was tested using parental and mCD1-transfected A20 cells (Fig. 3 B). mCD1$^+$ A20 cells can present both α-GalCer and the modified biotin–α-GalCer to the 3C3 hybridoma, whereas untransfected A20 cells could not. Other Vα14$^+$ NK T cell hybridomas could also recognize this compound (data not shown). Biotin-modified β-linked GalCer (biotin–β-GalCer) did not stimulate the T cell hybridoma (Fig. 3 B). These data indicate that the addition of a biotin group did not eliminate the ability of biotin–α-GalCer to be presented by mCD1 to NK T cells, nor did it cause β-GalCer to be recognized. However, it should be noted that the modification of the acyl chain of this compound, including the presence of the biotin group and two amide bonds, makes it a weaker antigen. In dose–response experiments with mCD1$^+$ APCs, biotin–α-GalCer gave a 1.5–2-fold lower stimulation than the unmodified α-GalCer (data not shown). Similarly, in antigen presentation experiments using CD1d-coated plates, it stimulated approximately three- to fivefold lower IL-2 release when incubated with either mCD1 (Fig. 3 C) or hCD1d molecules (data not shown).

Binding of CD1d Molecules to α- and β-GalCer. The ability of mCD1 and hCD1d molecules to bind to biotin–α-GalCer was examined using SPR. In our experimental set-up, biotin-GalCer compounds were immobilized on flow cell 2 of the streptavidin biosensor chip. No ligand was immobilized on flow cell 1, which therefore provided a control surface that allowed monitoring refractive index changes and nonspecific protein sticking. After the coupling procedure and every protein injection, the active surface is regenerated by washing with a solution containing Triton X-100, which removes any noncovalently bound lipid or protein. Therefore, binding to the exposed acyl chains of tethered α-GalCer molecules, which should be relatively free of aggregates, can be directly measured. At neutral pH, soluble

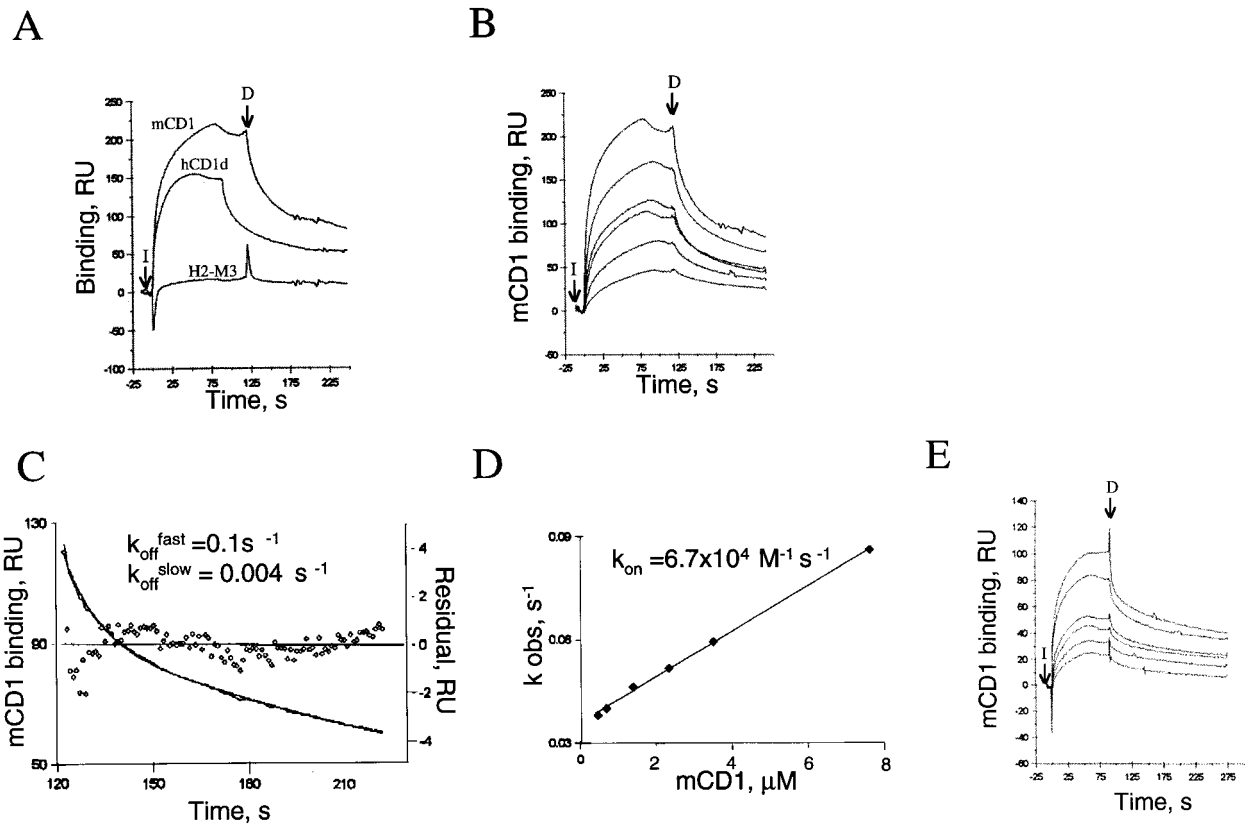

Figure 4. mCD1 and hCD1d can bind to immobilized biotin–α-GalCer and biotin–β-GalCer. (A) Binding of mCD1 (0.7 μM), hCD1d (0.5 μM), and H2-M3 (0.9 μM) to biotin–α-GalCer in PBS at 10 μl/min flow rate. I, injection; D, dissociation (washing of the chip in the running buffer). To control for refractive index changes, the RU values for the control flow cell (F_c1) have been subtracted from the RU values for the lipid-coupled flow cell (F_c2) to generate the sensograms shown. The noticeable dip in the binding curve during the last 20–25 s of the injection is due to the increased nonspecific sticking of the protein to flow cell 1, which generates a decreased binding signal in the subtracted ($F_c2 - F_c1$) sensogram. (B) Binding of 0.04, 0.06, 0.13, 0.22, 0.33, and 0.72 μM mCD1 to immobilized biotin–α-GalCer in PBS at 50 μl/min flow rate. (A and B) Representative data from one of three independent experiments. (C) Fitting the dissociation phase of mCD1 on biotin–α-GalCer surface to a double exponential model produces k_{off}^{fast} 0.1 s^{-1} and k_{off}^{slow} 0.004 s^{-1}. The contribution of the fast component is 22%. The plot includes observed data and fitted graph. Goodness of the fit is determined by the difference between the fitted values and the observed values (open symbols, plotted as Residual, RU). A residual RU with a value of less than the noise of the instrument (±3) indicates a good fit. (D) Association rate of mCD1 binding to biotin–α-GalCer surface. The slope of the plot of k_{obs} versus mCD1 concentration gives a k_{on} value of 6.7×10^4 $M^{-1}s^{-1}$. (E) Binding of 0.13, 0.16, 0.22, 0.33, 0.5, and 1.3 μM mCD1 to immobilized biotin–β-GalCer. All conditions were the same as in B.

mCD1 and hCD1d proteins, but not H2-M3, could bind immobilized biotin–α-GalCer (Fig. 4 A). The nonclassical class Ib molecule H2-M3 was chosen as a negative control, because, similar to mCD1, it has a hydrophobic antigen-binding groove (37). Additionally, soluble hCD1b protein was also able to bind to immobilized biotin-GalCer (data not shown). mCD1 binding to biotin–α-GalCer is dose dependent (Fig. 4 B), and similar dose–response data were obtained using hCD1d (data not shown). Binding of the plant lectins specific for the galactose portion of the immobilized GalCers (GSL I-B4 and RCA I) gave a signal similar in magnitude to the CD1d signal (29), suggesting that CD1d molecules bound to almost all of the glycosphingolipids on the chip that are accessible to macromolecules.

Fig. 4 C shows the dissociation curve of mCD1 from biotin–α-GalCer. The data can be best fitted to a two-component dissociation model with k_{off}^{fast} 0.1 s^{-1} and k_{off}^{slow} 0.004 s^{-1}, which correspond to half-lives of 7 s and 3 min, respectively. In different binding experiments, the faster component contribution varied between 20 and 30%. We estimated the association rate for mCD1 binding to α-GalCer from a single site model, plotting apparent association rate constant k_{obs} versus the concentration (Fig. 4 D). In different experiments, k_{on} values between 5 and 13 $\times$ 10^4 $M^{-1}s^{-1}$ were observed. By taking the weighed average of the dissociation rate (20% $\times$ 0.1 + 80% $\times$ 0.004 = 0.023 s^{-1}), we obtain a K_D of 0.34 μM (with the range of 5.9 $\times$ 10^{-8} M for the slow component of dissociation to 1.5 $\times$ 10^{-6} M for the fast component of dissociation). The calculated rate constants for hCD1d binding to α-GalCer are essentially equal to those obtained for mCD1 (Table I).

It has been speculated that the α-anomeric form of the sugar is essential for contacting the invariant NK T cell TCR, rather than for CD1d binding (18–23). Consistent with this hypothesis, both mCD1 and hCD1d could bind strongly to biotin–β-GalCer (Fig. 4 E, and Table I). The binding of β-GalCer to either mCD1 or hCD1d was surprisingly similar to the binding of α-GalCer, with respect to the two-component dissociation and the rate constants for association and dissociation (Table I). However, in several experiments the absolute number of RU for binding of either CD1d molecule to the β-GalCer–coupled sensor chips tended to be two- to threefold lower than for binding to α-GalCer–coupled sensor chips containing approximately the same number of RU of coupled compound (compare Fig. 4, B and E). Thus, within the limitations of our experimental set-up, it is still possible that there might be a two-

Table I. *Kinetic Analyses of the Binding of Immobilized Lipids to Human and Mouse CD1d*

CD1	Ligand	k_{on}	k_{off} (fast)	k_{off} (slow)
		$M^{-1}s^{-1}$	s^{-1}	s^{-1}
Mouse	α-GalCer	6.3×10^4	0.1	4×10^{-3}
Human	α-GalCer	1×10^5	0.08	4×10^{-3}
Mouse	β-GalCer	6.8×10^4	0.11	3×10^{-3}
Human	β-GalCer	6×10^4	0.12	3×10^{-3}
Mouse	DPPE	1.1×10^3	–	3×10^{-4}
Human	DPPE	1.4×10^3	–	2.7×10^{-4}

Data are representative of three experiments.

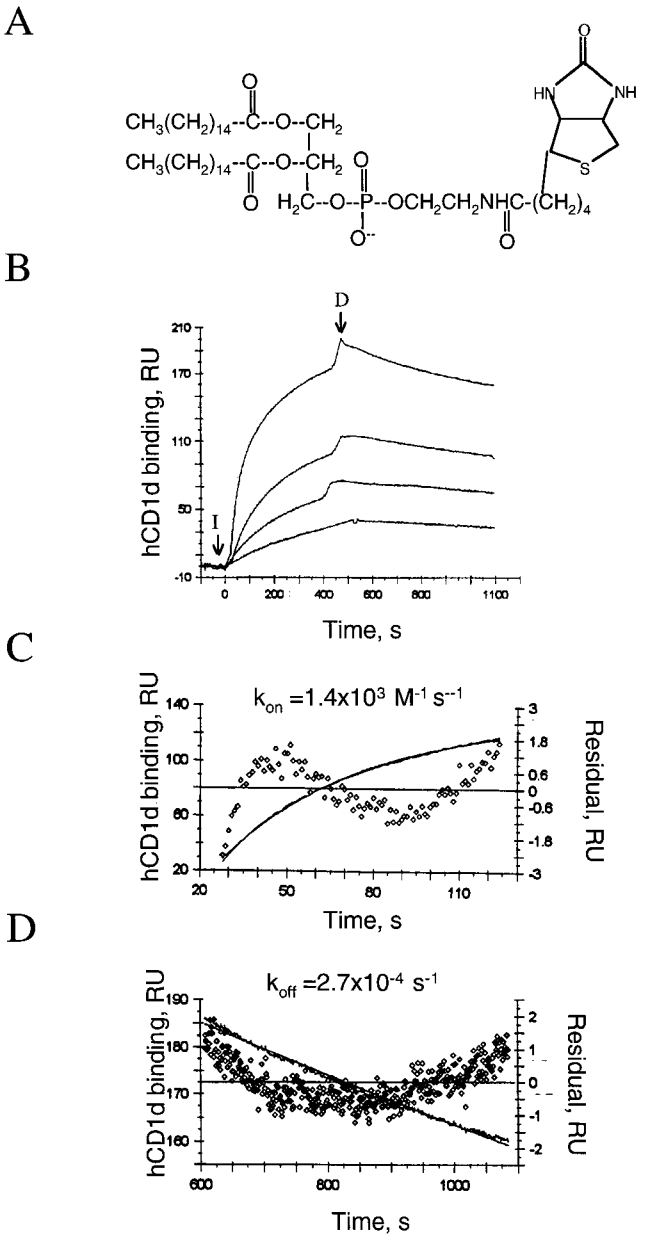

Figure 5. hCD1d binds to immobilized biotin-DPPE with slow association and slow dissociation. (A) Structure of *N*-(biotinoyl)dipalmitoyl-l-α-phosphatidylethanolamine (biotin-DPPE). (B) Binding of 1.4, 4, 5.6, and 14 μM hCD1d to immobilized biotin-DPPE in PBS (0.005% Tween) at 2 μl/min flow rate. (C) Fitting the association phase of hCD1d on biotin-DPPE surface gives a k_{on} value of 1.4 $\times$ 10^3 $M^{-1}s^{-1}$. The plot includes observed data and fitted graph. Goodness of the fit is determined by the difference between the fitted values and the observed values (plotted as Residual, RU). (D) Fitting the dissociation phase of hCD1d on biotin-DPPE surface to a single exponential model produces k_{off} 2.7 $\times$ 10^{-4} s^{-1}.

to threefold difference in affinity between β-GalCer and α-GalCer. However, because there is a much greater difference in the ability of β- and α-linked compounds to stimulate mouse NK T cells, we conclude that the lack of antigenicity of β-GalCer for NK T cells is due mainly to its failure to properly contact the invariant Vα14-containing TCR expressed by these cells, as opposed to a drastically reduced binding affinity for CD1d.

CD1d Binds to Phospholipids. To determine if CD1d can bind to phospholipids, as suggested by previous studies (26, 27), we used a different biotinylated lipid, biotin-DPPE (Fig. 5 A). This compound also permitted us to address the effect of having two unmodified acyl chains available for CD1d binding. Although the structure of the lipid portion of biotin-DPPE is quite different from that of galactosyl ceramides, and it is not antigenic for NK T cell hybridomas (data not shown), it is able to bind to CD1d proteins (Fig. 5 B). This binding is characterized by slower association, k_{on} 1.4×10^3 $M^{-1}s^{-1}$ (Fig. 5 C), and significantly slower dissociation, k_{off} 2.7×10^{-4} s^{-1} (Fig. 5 D), than binding to immobilized biotin–α-GalCer. These on and off rates produce a calculated K_D of 0.2 μM and a half-life of the CD1-biotin–DPPE interaction of 40 min. Therefore, like human CD1b (38), CD1d molecules can bind to diverse lipoglycans, although for CD1d molecules, acidic pH is not required. Furthermore, while we cannot exclude a possible contribution of the phosphate group or some other portion of the biotin-DPPE to the increased stability of CD1d binding, it is most likely that availability of two uninterrupted acyl chains accounts for the increased half-life of the biotin-DPPE complexes with CD1d. It probably also accounts for the slower association rate, as interactions between the two hydrophobic acyl chains may interfere with their loading into the separate A′ and F′ pockets of the CD1d antigen-binding groove.

Highly Glycosylated Sphingolipids Can Compete for CD1d Binding to Immobilized Lipids. To further demonstrate the specificity of biotinylated sphingolipid binding to soluble CD1d molecules, competition assays were carried out in which CD1d molecules were preincubated in solution with gangliosides, which are highly glycosylated sphingolipids. Gangliosides are not antigenic for the NK T cell hybridomas (data not shown), presumably on account of the β-linkage of the lipid-proximal sugar to the one carbon of the sphingosine. However, in the micromolar concentration range, gangliosides GM1 (five sugars) and GD1a (six sugars) could inhibit mCD1 or hCD1d binding either to biotin–α-GalCer or to biotin–β-GalCer. Representative data are shown for the inhibition by GM1 of mCD1 and hCD1d binding to biotin–α-GalCer in Fig. 6 A. Consistent with this finding, GM1 and GD1a could inhibit presentation of α-GalCer by either plate-bound mCD1 (Fig. 6 B) or hCD1d proteins (data not shown). A control hexasaccharide, disialyllacto-*N*-tetraose, did not inhibit presentation. This result indicates the importance of the ceramide portion of gangliosides for the competition for α-GalCer

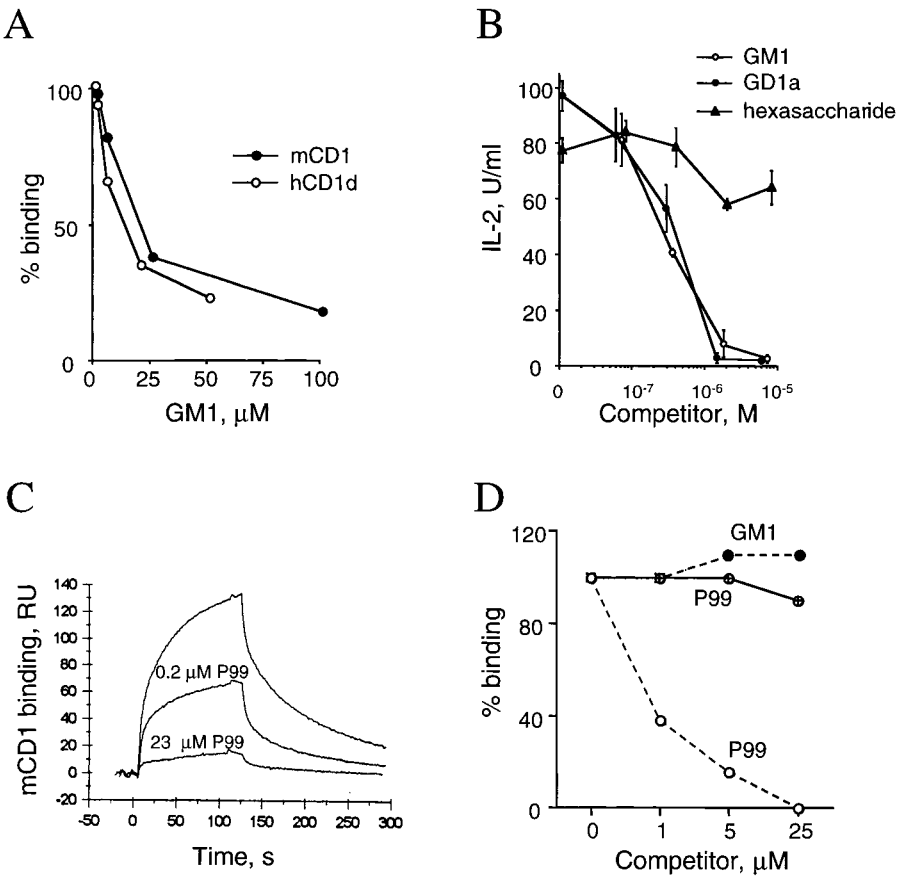

Figure 6. mCD1 can bind to both peptide and glycolipid ligands. (A) GM1 inhibits binding of mCD1 (1.8 μM) and hCD1d (1 μM) to the biotin–α-GalCer chip. Data are representative of three independent experiments. (B) Gangliosides GM1 and GD1a inhibit α-GalCer presentation by plate-bound mCD1 to the 3C3 hybridoma, whereas the control hexasaccharide has no effect. 1 μg/well (0.2 μM) mCD1 was preincubated with 25 ng/well α-GalCer (0.3 μM), with or without indicated concentrations of competitors. (C) Binding of 2 μM mCD1 to immobilized biotin-P99 peptide, and competition by 0.2 and 23 μM soluble P99 peptide. All conditions were the same as in the legend to Fig. 4 B. (D) mCD1 (1.8 μM) binding to the biotin-P99 chip (dashed lines) and to the biotin–α-GalCer chip (solid line). Biotin-P99 binding is inhibited by increasing concentrations of free P99 but not by GM1. P99 does not affect mCD1 binding to biotin–α-GalCer. All data shown are representative of at least three experiments in each case.

presentation. However, we cannot accurately estimate affinity constants from the CD1d–ganglioside interaction from these competition experiments because in the micromolar range, gangliosides are mostly in micelles (39), with very few monomers present. The inhibition by the gangliosides is most likely not due to the trapping of CD1d molecules in micelles, because CD1d molecules incubated with the gangliosides bound as well to an anti-CD1d mAb coupled to a biosensor chip as did the control CD1d molecules (data not shown).

Sphingolipid compounds containing only a single sugar, such as α-GalCer or β-GalCer, are much less soluble than the gangliosides, and unlike the gangliosides, must be dissolved in DMSO-containing solutions. Because of their insolubility and the adverse effect of the DMSO upon CD1d protein stability, we could not set up a reliable competition assay using α- or β-GalCer mixed in aqueous solution to compete for CD1d binding to the immobilized lipid ligands. From these data, we infer that the increased carbohydrate content of gangliosides, leading to increased solubility in aqueous solutions and therefore a higher rate of monomer formation, allows the similar lipid portions of these molecules to compete effectively for immobilized biotin-GalCer binding. Consistent with this hypothesis, we found that ceramide coupled to a highly polar polyethylene glycol moiety (PEG-ceramide) could also compete for binding to immobilized biotin–α-GalCer (data not shown). These data also underscore the importance of the ceramide group, and demonstrate that the oligosaccharide chain of the glycosphingolipid is not required for competition for CD1d binding.

Noncompetitive Binding of Peptides and Lipids to mCD1. We and others have shown that mCD1 is able to bind long hydrophobic peptides and present them to $CD8^+$ T cells (28, 40, 41). Using equilibrium binding studies, with radiolabeled 22-mer P99 peptide and mCD1 in solution, Castaño and colleagues (28) estimated the mCD1 affinity for antigenic peptide ligands to be ~0.9 μM, with fast association and dissociation kinetics. We examined mCD1 binding to immobilized biotinylated P99 peptide in the BIAcore (Fig. 6 C, and data not shown) and observed an association rate of $1–4 \times 10^4\ M^{-1}s^{-1}$ and a dissociation rate of $0.005–0.008\ s^{-1}$. These rates produce a calculated K_D in the range of 0.2–0.8 μM, a value in good agreement with equilibrium binding studies. The mCD1 binding to biotin-P99 was readily competed by free P99 peptide (Fig. 6, C and D). Similar to the results from peptide–MHC classical class I binding studies (42), a peptide concentration severalfold lower than total protein concentration resulted in a 50% inhibition of mCD1 binding. This suggests that only a percentage of the mCD1 molecules in our preparations are capable of binding the peptide, either because of the conformational state of the CD1d molecules, or because of some other factors such as differential occupancy of CD1d with lipid ligands. The binding of CD1d to immobilized P99 could not be inhibited by GM1 (Fig. 6 D), suggesting that GM1 and peptide do not bind to the same sites. Consistent with this, mCD1 binding to immobilized α-GalCer was not affected by soluble P99 peptide (solid line, Fig. 6 D) at concentrations that completely inhibited binding to immobilized peptide (dashed line, Fig. 6 D).

Discussion

In recent years, data have been generated on lipid antigen presentation by several different CD1 molecules (for reviews, see references 13, 43, and 44), but there is still very little information on the biochemistry of lipid antigen binding to CD1. The published crystallographic structure of mCD1 shows a deep, narrow, and very hydrophobic ligand-binding groove with two pockets termed A′ and F′ (45). This analysis also shows an acyl chain containing ligand inserted into the pockets of this groove (Degano, M., and I. Wilson, personal communication). Mass spectrometric characterization of the material eluted from mCD1 molecules purified from mammalian cell lines indicated the presence of a predominant GPI ligand (26). Our in vitro binding studies have demonstrated binding at acidic pH of phosphatidylinositol-based compounds in solution to an hCD1b-coupled chip in the biosensor (38). However, the signal obtained in these studies is suggestive of multimeric antigen binding to the immobilized CD1b molecules, and it remains to be determined whether the type of binding we measured corresponds to the formation of antigenic glycolipid–hCD1b complexes.

Here we have investigated the biochemistry of lipid antigen binding to the group II molecules hCD1d and mCD1, focusing on glycosphingolipids. These two CD1d molecules are of particular interest because they are recognized in a conserved manner by the NK T cell subset (24, 25). The ability of NK T cells to respond to CD1d is greatly augmented by the addition of α-GalCer, a compound isolated originally from the marine sponge *Agelas mauritanius*, based on its ability to prevent tumor metastases (46, 47). Glycosphingolipids in mammalian cells are considered to have only β-linked *dextro*-enantiomer sugar as the first sugar attached to ceramide. Compounds with an α-linked *dextro* sugar have not been found in mammalian cells, although it remains possible that low levels of such compounds are present but have not been detected. Regardless of whether α-GalCer is the natural ligand for NK T cells or merely cross-reactive with such a ligand, because of its ability to stimulate a large fraction of these cells and the availability of analogues (18–23), α-GalCer provides a very good model for studying the CD1–glycolipid antigen interaction.

Plate-bound CD1d molecules can present α-GalCer to NK T cell hybridomas. This demonstrates that some of the soluble CD1d molecules are in a native conformation, which is consistent with their ability to react with conformation-sensitive anti-CD1d antibodies. α-GalCer can be successfully loaded into the CD1d-binding groove at neutral pH, highlighting the differences in lipid binding between group I (CD1b) and group II (CD1d) molecules, at least in terms of acidification requirements for presentation. Although the loading of antigen onto CD1d molecules in acidified endosomes is not absolutely required for stable binding and antigen recognition (21), endosomal uptake augments the presenta-

tion of α-GalCer in some circumstances (18, 21). α-GalCer–CD1d complexes formed in vitro are physiologically relevant because they can be recognized by TCRs, formally proving that the presentation of α-GalCer is carried out by CD1d. We were surprised to find that some of the $V\alpha14^+$ T cell hybrids could react to a significant extent to the insect cell–derived plate-bound CD1d in the absence of exogenous ligand. Our results with soluble proteins provide one possible explanation for the CD1d autoreactivity of NK T cell hybridomas. At least some of them could be recognizing the CD1d protein directly, while the function of the endogenous or exogenous ligand could be to maintain CD1d molecules in proper conformation. Alternatively, it is possible that these T cells recognize the insect cell–derived ligand when bound to CD1d.

We have used immobilized, biotinylated forms of α-GalCer and β-GalCer to demonstrate specific binding to soluble mCD1 and hCD1d molecules. This has allowed us to obtain a more quantitative estimate of the affinity of lipid CD1 binding than was possible in a previous study, since in this case we examined the monomeric interaction between CD1 molecules and glycolipids. As determined by SPR studies, the affinity of both the mCD1 and hCD1d molecules for galactosyl ceramides and for phospholipids is in the 0.1–1 μM range. The near equivalence of α-GalCer and β-GalCer binding to both mCD1 and hCD1d is established by the results from multiple experiments. This provides the first evidence for the earlier prediction that the sugar linkage does not significantly affect CD1d binding (18) but is more likely to be important for TCR contact. SPR studies also indicate that the OH groups on the 3 and 4 positions of the sphingosine (29) are likewise not required for efficient CD1d binding (data not shown). Furthermore, the compounds that compete effectively for CD1d binding (Fig. 6 A) to biotin-GalCer include the gangliosides, which lack the OH on the 4 position of the sphingosine (see Fig. 3 C for sphingosine structure), and PEG-ceramide, lacking the hexose sugar entirely. Collectively, these data are consistent with the hypothesis that the CD1d molecule is primarily contacting the hydrophobic portions of the glycolipids. This idea is additionally supported by the finding that CD1d binds strongly to a phospholipid biotin-DPPE, which has a distinctly different polar head group. Based on these data, we conclude that CD1d molecules are likely to be capable of binding a variety of lipid-containing antigens.

The dissociation rate for CD1d binding to biotin–α-GalCer predicts a half-life of several minutes, faster than what was observed for MHC class I peptide dissociation (42, 48, 49), although there are some exceptions from SPR experiments, such as binding of Qa-1 protein to immobilized Qdm peptide (50). We believe that the relatively fast off rate for lipoglycan binding could be due to a combination of factors, including the method of attachment to the biosensor and structural changes in the biotinylated analogue itself. Indeed, SPR studies of MHC class I interaction with peptide have shown that the dissociation rate is significantly affected by both the amino acid through which the peptide is coupled to the sensor surface and the method of coupling (42). The sequestration of the terminal part of the acyl chain in the streptavidin-binding pocket will prevent the acyl chain from completely filling one of the two CD1d antigen-binding pockets. Furthermore, the presence of the two hydrophilic amide bonds in the acyl chain (Fig. 3 A) and the presence of the biotin group at the end of it create a suboptimal antigen (Fig. 3). Therefore, the CD1d interaction with immobilized biotin-GalCer is probably limited to a major contribution from the sphingosine only, leading to a faster off rate. This interpretation is supported by the longer lasting CD1d binding to immobilized biotin-DPPE, with two unmodified lipid chains that can insert into hydrophobic CD1d antigen-binding pockets.

The dissociation of the peptide P99 for CD1d also has a relatively fast off rate, similar to the off rate for CD1d binding to the immobilized sphingolipids. This is true for measurements of CD1d peptide binding made in solution previously (28) as well as for measurements made with peptide coupled to the biosensor chip through the NH_2-terminal biotin (Fig. 6) or through in an internal cysteine (data not shown). Because of these consistent findings, the dissociation is not likely to be due to modifications of the peptide or details of the experimental set-up. The fast off rate is consistent with the finding that peptide-reactive T cells require a relatively high dose of antigen, in the micromolar range, in order to be stimulated (28, 41). The existence of two very different kinds of mCD1-binding ligands presents a significant biological puzzle (43). Where in the mCD1 molecule can the two types of ligands be accommodated? The results from competition studies suggest that peptides and lipids do not compete for the same site. This is consistent with our earlier finding that neither P99 nor an ovalbumin peptide could prevent α-GalCer recognition by NK T cells, while α-GalCer did not affect ovalbumin peptide recognition by mCD1-restricted CTLs (41). Although there is evidence that lipid binds in the groove, we can only speculate about the mechanism for peptide binding. The possibilities include peptide binding outside the groove, or peptide binding to the groove of a subset of CD1d molecules. This peptide-binding CD1d subset might have a particular posttranslational modification, or it might require particular lipids in the groove, in which case the peptide might lie across the top of the groove bound to CD1d and the lipid.

In conclusion, using a system in which aggregation of the antigen is minimized, we have definitively established and quantified by SPR the interaction of CD1d or group II CD1 molecules with monomeric glycosphingolipids and phospholipids. We have also demonstrated that the orientation of the sugar on the glycosphingolipid is more important for TCR than for CD1d contact, and that CD1d binds primarily to the hydrophobic acyl chains. Unlike lipid binding to CD1b, these interactions can occur at neutral pH and lead to the formation of antigenic CD1d–lipid complexes in vitro. Much still remains to be learned concerning the means by which the hydrophobic acyl chains are made available for CD1d binding in cells, the structure of the CD1d–lipid antigen complex, and the mechanism of peptide binding to mCD1.

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